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Phenotypic and transcriptional responses associated with multi-generation exposure of *Folsomia candida* to engineered nanomaterials†

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Sublethal effects of toxicants may cumulate over time and become apparent only when test organisms are exposed for multiple generations. In this study we determined phenotypic effects and transcriptional responses in the parthenogenetic soil invertebrate *Folsomia candida* over four generations, followed by two generations of recovery. Animals were exposed to two metal-based nanomaterials (NMs): copper oxide (CuO) and tungsten carbide–cobalt (WCCo), both homogenously mixed in with the soil. Survival and reproduction were not affected in any of four consecutive generations of *F. candida* exposed to CuO-NM at concentrations as high as 6400 mg Cu per kg dry LUFA 2.2 soil. WCCo-NM affected reproduction and survival from the third generation onwards, with EC_{50} values between 2400 and 5600 mg NM per kg dry soil, but recovery was seen in recovery generations 1 and 2 when kept in clean soil. Histological investigations showed that WCCo-NM (3200 mg kg^{-1}) induced tissue damage and loss of villi from the gut epithelial cells. Expression of four target genes known to be responsive to stress were investigated by quantitative PCR at different exposure levels and in different generations. Expression of all genes was significantly affected by NMs even though exposures were below toxic threshold concentrations. In addition, gene expression did not always return to control levels during consecutive recovery over two generations in clean soil. This shows that gene expression assays can detect physiological alterations cumulating from one generation to the next initially without visible effects on phenotypic variables such as reproduction. The possibility of multi-generation carry-over of sublethal toxicity needs more attention in environmental risk assessment.

Environmental significance

Sublethal effects of nanomaterials may cumulate over time and become apparent only when soil organisms are exposed for multiple generations. Here, we study the phenotypic and transcriptional responses of the soil invertebrate *Folsomia candida* associated with multi-generation exposure to engineered nanomaterials. This manuscript is novel as it assesses responses at different levels of integration, by measuring reproduction, survival and gut epithelium integrity as well as gene expression profiles. We show that adverse effects of nanomaterial exposure can only be detected after exposure for subsequent generations, by observing induced gut tissue damage and a decrease in reproduction. Also, by examining gene expression assays, we detected physiological alterations in response to nanomaterial exposure even when consequences on reproduction were not observed.

1. Introduction

Increased production and application of engineered nanomaterials (NMs) inevitably leads to increased environmental release of such compounds. Hence, there are growing concerns about potential adverse effects on different organisms exposed to NMs.[1,2] NMs are manufactured particles smaller than 100 nm that are commonly used in everyday consumer, industrial and medical products due to their large surface area to mass ratio, catalytic capacity, antimicrobial activity and other characteristics.[3–5] Among the NMs gaining commercial and industrial use and popularity, copper-oxide (CuO) and tungsten (wolfram) carbide-cobalt (WCCo) have widespread uses in batteries, surface coatings and surgical instruments.[1,6,7] A number of studies have already described effects of CuO-NMs. For example, Adam *et al.* (2015) found that reproduction and body-size of *Daphnia magna* was

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affected (concentration reducing reproduction by 50%, EC_{50}, was 1041 mg Cu l^{-1}) after 21 days of exposure.[1] Gonçalves *et al.* (2017) described a shorter lifespan for the potworm *Enchytraeus crypticus*, where time to 50% mortality (LT50) decreased from 218 days in controls to 145 days in CuO-NM-exposed animals.[8] There is only a handful of studies describing the effects of WCCo-NM to environmental targets. For instance, Ding *et al.* (2009) found increased oxidative stress at concentrations ranging from 20 to 150 μg cm^{-2} when investigating mouse (*Mus musculus*) epidermal cells.[9] WCCo-NM toxicity was also reported in rainbow trout (*Oncorhynchus mykiss*), where a significant reduction in gill cell viability was found after three hours and three days of exposure (8.25–33 mg l^{-1}).[6]

Current ecotoxicological tests generally run for one generation or even shorter and do not take into account the long-term effects that could appear due to multigenerational exposure. Such effects include reproductive failure and bioaccumulation passed on to offspring, physiological changes leading to adaptation or acclimatization, or even extinction of populations.[10–13] Consequently, the number of studies describing multigenerational effects of nanomaterial exposure is currently increasing. For example, a study on *Caenorhabditis elegans* showed that reproduction decreased in an unexposed F2 generation when the parental generation was exposed to gold nanoparticles, but then slowly recovered again in generations F3 and F4.[11] The authors suggested that parental exposure to gold nanoparticles affected reproduction in unexposed subsequent generations through effects on the germ line. A recent study by Moon *et al.* (2017) also described multigeneration effects of gold nanoparticles following two types of exposure patterns (continuous and intermittent) in *C. elegans*.[13] The authors observed a decrease in reproduction and an increase of abnormalities in the reproductive organs of *C. elegans* from generations P0 to F4. The authors concluded that exposure time and historical factors should be taken into account when examining the effects of nanomaterials.

To get a better understanding of the mechanisms underlying physiological shifts in response to toxicants and about carry-over effects, gene expression and genomic tools are increasingly integrated in standardised ecotoxicology tests.[14] By using a number of target genes as biomarkers, potential adverse effects of NMs to organisms could be elucidated. In the present study, four target genes were used for gene expression assays: metallothionein, ABC transporter, IPNS and Laminin A. Metallothioneins are metal-binding proteins that play a role in the protection against metal toxicity and cellular redox disturbance.[15–17] ATP-binding cassette (ABC) transporters comprise a large gene family that facilitates the transport of solutes through membranes, enabled by adenosine triphosphate (ATP).[18] Each transporter is induced by a different spectrum of solutes, which is reflected in potentially differential gene expression among the ABC transporter gene family members. Isopenicilin N synthase (IPNS) is involved in the β-lactam antibiotics biosynthesis pathway that was shown to be horizontally transferred into *F. candida's* genome.[19] Several studies have shown that IPNS is highly inducible over a wide range of environmental stress factors *e.g.*,[20] implying that this gene is part of the general stress response of *F. candida*. Finally, laminins are proteins that are a major part of the basal lamina, influencing cell differentiation and migration.[21]

In the present study we provide, for the first time, a comprehensive analysis of multi-generation NM exposure effects to *F. candida*, including recovery generations. We studied *F. candida* exposed to either CuO or WCCo nanomaterials for four consecutive generations (exposed generations), plus an additional two generations in clean soil (recovery generations). We assessed responses at different levels of integration, by measuring reproduction, survival and gut epithelium integrity. We also examined gene expression profiles in order to investigate whether a potential carry-over effect from parental generation to subsequent generations may be present and to assess whether historical exposures and generation could cause a shift in physiological response to NMs by for example showing an increased sensitivity or adaptation to new exposures.

2. Materials and methods

2.1 Test compounds and spiking of soil

Loamy sand soil (LUFA 2.2) (Speyer, Germany) was used as the test soil. This soil has a reported total organic carbon content of 2.09%, pH_{CaCl_2} of 5.5, cation exchange capacity (CEC) of 10.0 $cmol_c$ kg^{-1} and a water holding capacity (WHC) of 46.5%.[22]

Tungsten carbide–cobalt (WCCo) NM and copper oxide (CuO) NM were provided by the Sustainable Nanotechnologies Project (SUN) and were purchased from different suppliers (for details see Table 1). As positive controls copper(II) chloride ($CuCl_2$) and cobalt chloride ($CoCl_2{\cdot}6H_2O$) were previously used and described by Noordhoek *et al.* (2018).[23] For WCCo-NM, nominal test concentrations were 0–200–400–800–1600–3200–6400 mg WCCo-NM per kg dry soil. CuO-NM were tested at nominal concentrations of 0–200–400–800–1600–3200–6400 mg Cu per kg dry soil. For each test concentration, the corresponding quantity of dry test compound was mixed in with the required amount of dry LUFA 2.2 soil. Subsequently, the soil was moistened with deionized water to 50% of the WHC and mixed once more to ensure a homogeneous distribution of the NMs in the soil. Finally, the spiked soil was divided over replicate test jars and allowed to equilibrate for 1 day before starting the exposures. *Folsomia candida* was exposed for four consecutive generations, using freshly spiked soil at the start of every new generation.

In a previous study we determined total metal concentrations in soil and in the pore water for CuO-NM and copper(II) chloride ($CuCl_2$) and for WCCo-NM and cobalt chloride ($CoCl_2{\cdot}6H_2O$).[23] Total metal (Cu, Co) concentrations in the test soil ranged between 88 and 130% of the added total concentrations for CuO-NM, $CuCl_2$ and $CoCl_2$. LUFA 2.2 control

Table 1 Characteristics of the nanomaterials tested for their multi-generational toxicity to *Folsomia candida*

Compound	Particle size (BET) (nm)	Surface area (BET) (m^2 g^{-1})	Surface by volume (m^2 cm^{-3})	Skeletal density (g cm^{-3})	Physical state	Appearance	Shape	CAS number	Supplier
WCCo	67	7.01	89	12.74	Powder	Solid	NA	12070-12-1 (WC) 744-48-4 (Co)	MBN
CuO	15–20	47	NA	6.3	Powder	Solid	Spherical	1317-38-0	PlasmaChem

BET = Brunauer, Emmett and Teller particle size and surface area analysis. NA = information not available. WCCo = tungsten carbide–cobalt, CuO = copper oxide.

soil contained on average 0.83 and 1.5 mg Co per kg dry soil (WCCo-NM and $CoCl_2$ test, respectively) and 4.4 and 4.7 mg Cu per kg dry soil (CuO-NM and $CuCl_2$ test, respectively). On average 7% of the nominal WCCo-NM concentration added was retrieved as Co in soil, which corresponds to the manufacturers' information.

2.2 Multi-generation toxicity tests

We used the parthenogenetic springtail *Folsomia candida* (Hexapoda, Collembola, Isotomidae, "Denmark strain", VU Amsterdam) as a model organism. Cultures were kept in a climate room at 16 ± 0.5 °C, a light intensity of 2343 Lux and a 16/8 h light/dark regime. To obtain synchronized animals, mature adults were allowed to lay eggs in plastic containers with a moist bottom of plaster of Paris (consisting of gypsum and charcoal) for two days at 20 ± 0.5 °C. The first generation was started with 10–12 day old juveniles hatched from these eggs. All toxicity tests were performed in a climate room at 20 ± 0.5 °C, 75% relative humidity, a light intensity of 2343 Lux and a 16/8 h light/dark regime. Each experiment lasted 30 days instead of the standard 28 day toxicity test as described in OECD guideline 232.[24] This was done to obtain sufficient juveniles of approximately 10–12 days old to start the toxicity test for the next generation. For every generation tested, 100 ml glass jars were used and five replicates were prepared for each concentration and control. Ten animals were introduced into each test jar with 30 grams of moist soil and a few grains of dry baker's yeast (instant yeast from Algist Bruggeman N. V, Ghent, Belgium). Each jar was closed with a bakelite screw top. Once a week jars were aerated, moisture loss was replenished with deionized water and animals were fed. For every generation we extracted animals after 30 days from soil by adding 100 ml of deionized water to each test jar, gently stirring and transferring them to a plastic beaker, allowing all springtails to float on the surface. Pictures were taken to later count all animals with the software program ImageJ to determine survival (number of adults) and reproduction (number of juveniles). The next generation was started by randomly picking five times ten animals from all offspring collected per exposure concentration; these were used to inoculate five replicates with ten animals each, and exposed to the same exposure concentration.

In this manner, *F. candida* and its offspring were exposed to CuO and WCCo NMs for four consecutive generations. For every generation, LUFA 2.2 soil was freshly spiked with either CuO or WCCo NMs. For the fifth and the sixth generation animals were transferred to clean soil, to find out whether there were any carry-over effects from previously exposed generations to non-exposed animals.

2.3 Histology

Since the gut is the springtail's main metabolic organ we looked at integrity of the gut epithelium, as a more physiological endpoint. In a previous study we already observed a decreasing trend in reproduction in WCCo-NM exposed *F. candida* whereas such a trend could not be observed from CuO-NM exposed animals. Therefore, for our histological analyses we decided to examine animals exposed for four subsequent generations to WCCo-NMs. Three surviving springtails for four generations per treatment (from control and high (3200 mg WCCo-NM per kg) concentrations) were fixed into a glass vial containing Bouin's solution (4 ml of formaldehyde 40%, 15 ml of saturated picric acid in water and 1 ml of acetic acid). After fixation, fixative solutions were removed by aspiration from each sample, and springtails were washed and stored in 70% ethanol (2 mL) at 4 °C. Samples were treated twice for 30 min with increasing percentages of ethanol using increments of 10% up to 100%. Then, samples were treated two times for 30 min with ethanol : amyl acetate in a 1 : 1 ratio, followed by amyl acetate : paraffin (1 : 1) treatment for 30 min. Finally, samples were kept for 60 min in 100% paraffin at 60 °C and embedded in paraffin blocks at room temperature. Serial longitudinal sections (7 μm thick) were cut using a microtome (HM 355S, Microm Internation GmbH). Sections were serially placed on microscope slides, and three concurrent staining protocols were used: 1) hematoxylin and eosin; 2) Mayer's hematoxylin solution (Sigma) which colours the cell nucleus blue; and 3) eosin Y solution, alcoholic (Sigma), which gives a red colour to the cytoplasm. All stained *F. candida* longitudinal sections were visually inspected using an Olympus CX41 microscope, and photographs were captured using an Altra 20 soft imaging microscope camera (Olympus) with 40× objective.

2.4 RNA extraction

To investigate the physiological shifts in response to NMs or a potential carry-over effect, the effects of historical treatment ("History") and generation ("Generation") on new/current

treatment ("Exposure") were examined in the parthenogenetic springtail *Folsomia candida* ("Denmark strain", VU Amsterdam) by performing gene expression tests.

We examined the gene expression profiles of animals after three exposed generations and from animals after two recovery generations, all originating from previously unexposed controls, a low (800 mg kg^{-1}) and a high (3200 mg kg^{-1}) concentration of either CuO or WCCo NMs. Note that the 'recovery animals' that were now exposed to low and high exposures had already been in a recovery stage for two generations, during which the animals did not encounter any NM exposure. Similar to the multi-generation toxicity tests, animals were collected when they were approximately 12 days old. Subsequently, all animals were placed on clean soil for 8 days allowing them to become approximately 20 days old, which is the standardized age of *F. candida* in gene expression analyses. Pools of 30–50 animals were then exposed for 2 days (new/current exposure) to each of the nanomaterials in jars containing 30 grams of either clean soil or polluted LUFA 2.2 soil, using five replicates per treatment (Fig. S1†). Animals were then extracted and deposited in microcentrifuge tubes to be snap frozen with liquid nitrogen. Samples were kept at −80 °C prior to RNA isolation. Total RNA was extracted using the SV Total RNA Isolation System (Promega, USA), following the manufacturers protocol. RNA quantity and purity were measured with a NanoDrop ND-1000 Spectrophotometer.

2.5 qRT-PCR analysis

Previously designed primer sets[17] were applied in Q-PCR to quantify expression of four target genes: metallothionein-like motif containing protein (MTC, Fcan01_08822-PA), ATP-binding cassette transporter (ABC, Fcan01_27073), isopenicillin N synthase (IPNS, Fcan01_27072) and laminin alpha (LAM A, Fcan01_06635) and two endogenous reference genes tyrosine 3-monooxygenase (YWHAZ, Fcan01_06830) and succinate dehydrogenase (SDHA, Fcan01_08383) (Table 2), all having an annealing temperature of 60 °C and amplicon length of 80–120 bp with 45–55% GC content.

Approximately 2 μl of RNA (between 10 to and 40 ng μl^{-1}) input per sample was used for reverse transcription using 200 U M-MLV reverse transcriptase (Promega, USA). Subsequently, cDNA was diluted 1 : 5 and 2 μl was used in 20 μl PCR reaction volumes containing SensiMix™ Sybr® No-ROX master mix (Bioline, UK) with 0.25 μM of each of the forward and reverse primers. The qPCR reactions were performed in duplicate for each sample using a CFX Connect Real Time PCR Detection system (BIO-RAD, USA) with universal conditions (10 min at 95 °C, 15 s at 95 °C, 1 min 60 °C, 40 cycles).

2.6 Data analysis

Effect concentrations that reduced survival and reproduction by 50% compared to untreated controls (LC_{50} and EC_{50}, respectively) were determined using a logistic dose–response model; 95% confidence intervals for the estimates were calculated by nonlinear regression analysis in IBM SPSS Statistics 23 software.[25] One-way analysis of variance (ANOVA) followed by Dunnett's *post hoc* test ($P < 0.05$) was used to test for differences between controls and treatments. All effects are expressed on the basis of nominal test concentrations.

Gene expression levels were normalized by including two reference genes (YWHAZ & SDHA) in the analysis.[26] Normalized gene expression was calculated using BioRad CFX Manager software (version 3.1) by dividing the expression level of the target gene by the average expression of the two reference genes, while correcting for amplification efficiency. The mean normalized expression levels were $\ln(x + 1)$ transformed prior to subsequent analysis to fulfil the assumptions for normality and homogeneity of variance. This transformation was sufficient for all but three gene expressions: the MTC gene in both CuO and WCCo NM exposed animals, as well as the IPNS gene in WCCo-NM exposed animals, which were thus analyzed by using a generalized linear model with quasi Poisson distribution. Significance of altered gene expression was assessed by fitting linear models for normalized gene expression with "Exposure", "History", and "Generation" as fixed factors. All interactions between fixed factors were included in the model. Consequently, a three-way ANOVA revealed which interactions and main factors significantly contributed to the fit of this model. Contrasts analysis revealed which levels within the significant factors differed from each other.

Table 2 Gene markers and primer sequences for Q-PCR assays with *Folsomia candida* exposed to CuO or WCCo nanomaterials in LUFA 2.2 soil

Biomarkers	Function	Primer efficiency (%)	Sequence
Fcan01_06830	YWHAZ	93.2	Forward: TCGCCCTCAACTTTTCCGTT Reverse: TGCTATCGCTTCATCGAATGCT
Fcan01_08383	SDHA	98.6	Forward: ACACTTTCCAGCAATGCAGGAG Reverse: TTTTCAGCCTCAAATCGGCA
Fcan01_08822-PA	Metallothionein like motif containing protein	96.2	Forward: AGCCAATATTTTCGAGTGGAGA Reverse: CAAGATGCTCGAATAGCAACAGTA
Fcan01_27073	ABC transporter	90.6	Forward: GTGTGAAATCTGGCGAAAAGGT Reverse: TTGAGCAGCAGAAGGCACTAATC
Fcan01_27072	Isopenicillin N synthetase	84.4	Forward: GACATGTCGGCAAAACTCCTTC Reverse: GGGTAGCGAATAAGTCGCACTG
Fcan01_06635	Laminin A	94.5	Forward: AAATGTTGTGAGAGTGGAGCAGG Reverse: CTTGGATTTAACTCCGTCGCAT

Finally, we tested for correlations between gene expression patterns for each compound using a Pearson's correlation test. All statistical analyses on gene expression data were performed using R software (R Core Team 2014, version 3.1.2) in RStudio (RStudio Team 2016, version 1.0.136).

3. Results

3.1 Toxicity of CuO and WCCo nanomaterials

Most toxicity tests met the OECD validity guidelines, obtaining >80% adult survival, on average >100 juveniles per replicate test jar by the end of the test, and a coefficient of variance (CV) <30% of the mean number of juveniles in the untreated controls. Survival of *F. candida* in LUFA 2.2 soil spiked with up to 6400 mg Cu per kg dry soil of CuO nanomaterial was not significantly affected for any of the four generations exposed and subsequent two generations in clean soil (Fig. 1 and 2). Control animals showed increased survival with subsequent generations (*i.e.* 74%, 92%, 88%, 84%, 96% and 100%, respectively). Untreated control animals in the WCCo-NM experiment showed >80% survival in all six generations (*i.e.* 90%, 84%, 100%, 85%, 98% and 100%, respectively). Survival of *F. candida* in LUFA 2.2 soil spiked with up to 6400 mg WCCo-NM per kg dry soil was not significantly affected during the four generations in treated soil and recovery generation 2 in clean soil, and was comparable to the controls (89%, 90%, 91%, 87% and 92%). In recovery generation 1 (in clean soil), survival was significantly decreased (F = 4.7, p = 0.001) for animals that were previously (generations 1–4) exposed to 6400 mg WCCo-NM per kg dry soil.

On average, 870 juveniles (coefficient of variance (CV) 15%) were found in controls of exposed generation 1 from the CuO-NM test. For subsequent generations (exposed generations 2, 3, 4 and recovery generations 1 and 2) we found on average 2147 (CV 47%), 1789 (CV 36%), 1163 (CV 31%), 696 (CV 6%) and 2739 (CV 16%) juveniles per container, respectively. Although the mean number of juveniles in the exposed generation 1 and recovery generation 1 were considerably lower compared to other generations, reproduction was not affected at concentrations up to 6400 mg Cu per kg dry soil in any of the generations from the CuO-NM test (Fig. 1 and 2).

In the controls from WCCo-NM exposed generations 1 to 4 and recovery generations 1 and 2 we found on average 1555 (CV 22%), 1382 (CV 31%), 1445 (CV 27%), 1642 (CV 55%), 988 (CV 22%) and 2566 (CV 9%) juveniles, respectively. Exposure to WCCo-NMs decreased *F. candida* reproduction in a dose-dependent manner (Fig. 3) in exposed generations 3 and 4 with EC_{50} values of 2463 (95% CI 945–3981) and 3836 (2682–4989) mg NM per kg dry soil, respectively. For recovery generation 1 (kept in clean soil) an EC_{50} value of 5658 (CI could not be calculated) mg NM per kg dry soil could be estimated, however, the difference with the control was significant only at 6400 mg WCCo-NM per kg dry soil (F = 8.62, $p < 0.001$) (Fig. 4). According to a likelihood-ratio test, EC_{50} values of exposed generations 3 and 4, and recovery

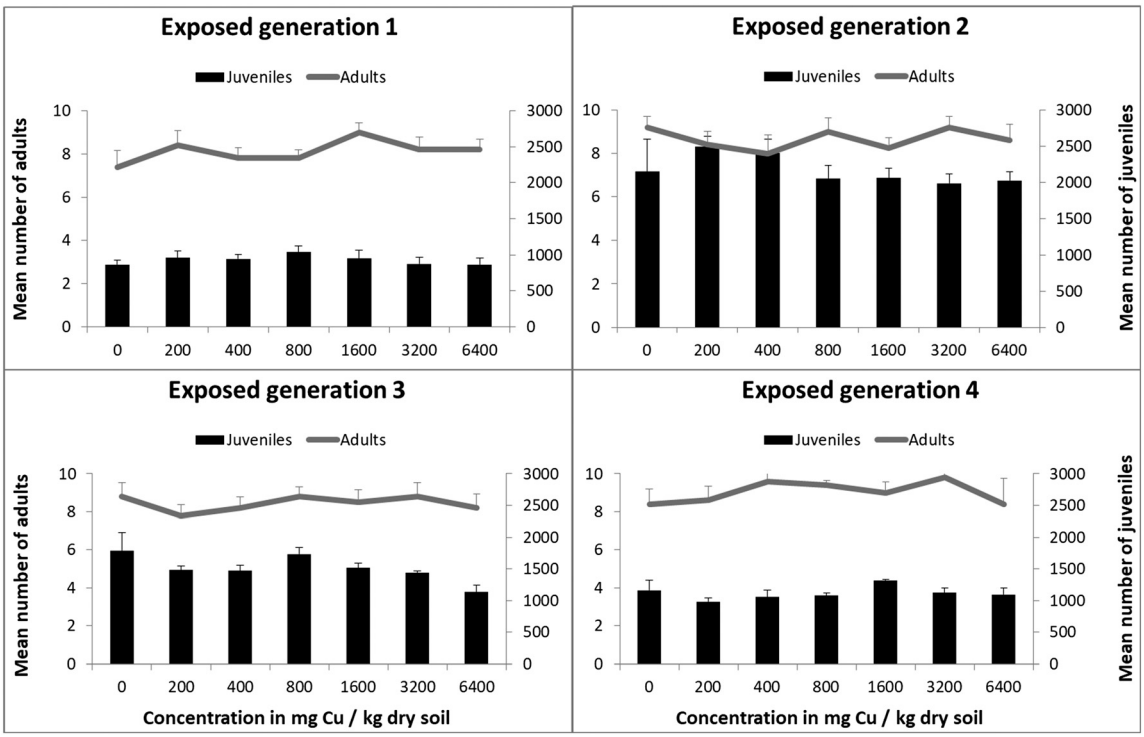

Fig. 1 Effect of CuO nanomaterials on the survival and reproduction (number of juveniles per container) of *Folsomia candida* after 1, 2, 3 and 4 generations of exposure in LUFA 2.2 soil. Nominal Cu concentrations in the soil are provided on the *x*-axis. Mean number of surviving adults is provided on the left *y*-axis and mean number of juveniles produced on the right *y*-axis.

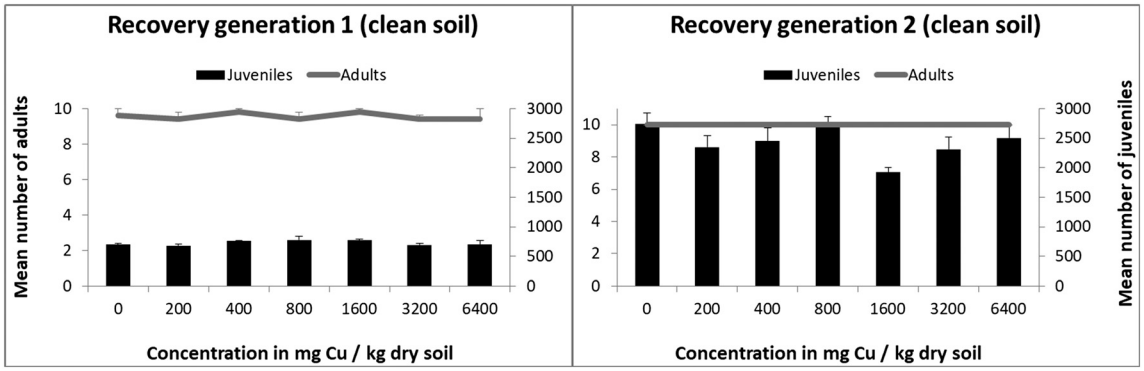

Fig. 2 Effect of CuO nanomaterials on the survival and reproduction (number of juveniles) of *Folsomia candida* recovery generations 1 and 2 (*i.e.* in their recovery phase in clean LUFA 2.2 soil). Nominal Cu concentrations during the previous four generations exposed to CuO-NM are provided on the *x*-axis. The mean number of surviving adults is provided on the left *y*-axis and the mean number of juveniles produced on the right *y*-axis.

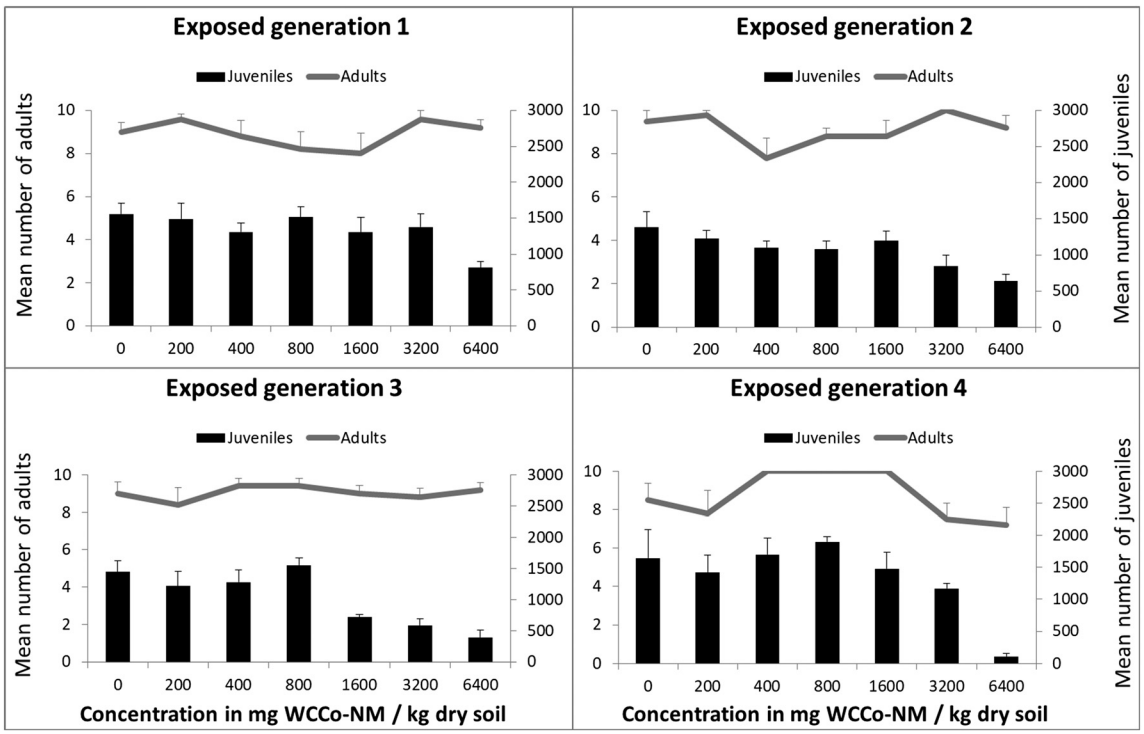

Fig. 3 Effect of WCCo nanomaterials on the survival and reproduction (number of juveniles) of *Folsomia candida* after 1, 2, 3 and 4 generations of exposure in LUFA 2.2 soil. Nominal NM concentrations in the soil are provided on the *x*-axis. Mean number of surviving adults is provided on the left *y*-axis and mean number of juveniles produced on the right *y*-axis.

generation 1 did not differ significantly from each other ($\chi_{2(1)}$ = 0.06 to 1.56).

3.2 Histological observations

High WCCo-NM exposure concentrations (3200 mg NM per kg dry soil) induced tissue damage in *F. candida*. Fig. 5 shows that abnormal morphology of the gut epithelium was observed after four generations of WCCo-NM exposure. Intact microvilli were observed on the apical side of all epithelial cells (arrow), while in gut tissue from animals exposed to the high WCCo-NM concentration these microvilli were degenerated, indicating serious damage to the nutrient absorption function of the gut.

3.3 Effects of CuO and WCCo nanomaterials on gene expression

To investigate potential carry-over effects and physiological shifts in stress response, expression of MTC, ABC transporter, IPNS and Laminin A (Fig. 6 and 7) in response to new/current treatments (control, 800 mg kg^{-1} and 3200 mg kg^{-1}) was

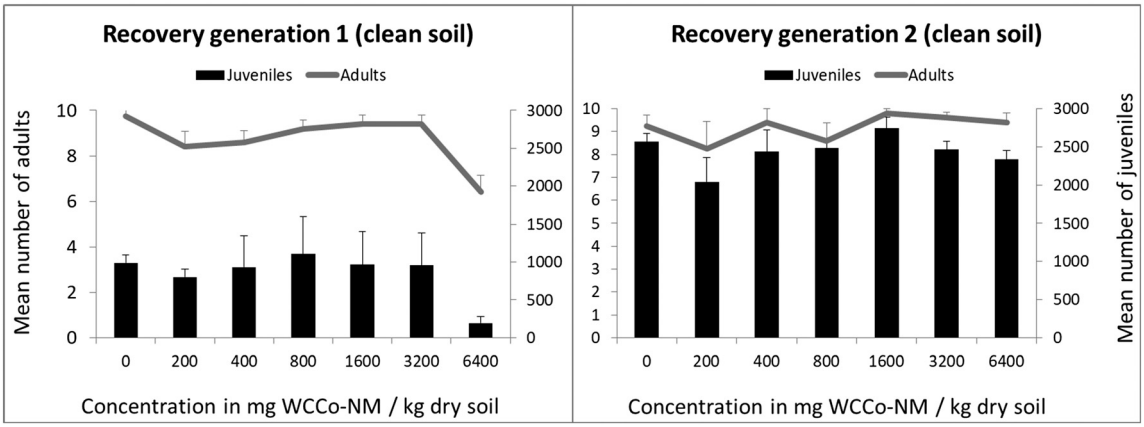

Fig. 4 Effect of WCCo nanomaterials on the survival and reproduction (number of juveniles) of *Folsomia candida* in recovery generations 1 and 2 (*i.e.* in their recovery phase in clean LUFA 2.2 soil). Nominal NM concentrations during the previous four generations exposed to the WCCo NM are provided on the *x*-axis. The mean number of surviving adults is provided on the left *y*-axis and the mean number of juveniles produced on the right *y*-axis.

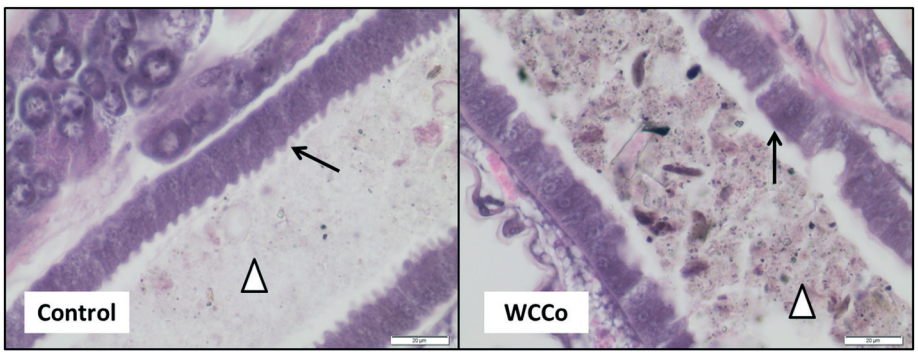

Fig. 5 Histological longitudinal sections of *Folsomia candida* stained with hematoxyline-eosine (HE). Left panel: Section of the intestine of a 4th generation control individual. Right panel: Section of the intestine of an individual from a population exposed for 4 generations to a high (3200 mg kg^{-1}) concentration of WCCo-NM. Triangles indicate the gut lumen, arrows indicate presence (control) and absence (WCCo) of microvilli.

assessed in animals after three exposed generations and after two recovery generations originating from previously unexposed controls, a low (800 mg kg^{-1}) and a high (3200 mg kg^{-1}) concentration of either CuO or WCCo NMs.

We found a significant effect of CuO-NM exposure on MTC gene expression ($p < 0.0001$) (Table 3). Fig. 6 indicates that MTC expression increased when animals were exposed to the highest current CuO-NM concentration (3200 mg kg^{-1}), while animals that have a high CuO-NM exposure history show increased MTC expression regardless of the current exposure level. Interestingly, this pattern is completely reversed after two recovery generations. Three-way analysis of variance (ANOVA) also revealed significant effects for CuO-NM exposure in IPNS ($F = 22.08$; $F(df) = 2$; $p < 0.0001$) and Laminin A ($F = 11.47$; $F(df) = 2$; $p = 0.0001$) (Fig. 6) (Table 3). IPNS expression increased with increasing current CuO-NM exposure concentration. This pattern was also observed in the recovery phase. In case of Laminin A, significant interactions could be observed between historical exposure and new exposure ($F = 3.25$; $F(df) = 4$; $p = 0.02$) (Table 3). For instance, Laminin A expression significantly differed between 'control → 0' and 'control → 3200' ($p = 0.003$) with an increased expression in the latter, where in both cases the historical exposure treatment was 'control' and the new exposure treatment was '0' and '3200', respectively. Also, significant interactions were observed between 'control → 0' and 'low → 3200' ($p = 0.005$) and between 'control → 0' and 'high → 800' ($p = 0.001$). Furthermore, contrasts analysis on the interaction History*Generation revealed that control animals (History: control; Exposure: 0) after three exposed generations had a significantly different gene expression of Laminin A compared to all three historical treatments (control, low and high) in animals after two recovery generations ($F = 5.18$; $F(df) = 2$; $p = 0.01$) (Table 3), with the latter being all significantly upregulated compared to the control animals (control → 0) after three exposed generations. There was a strong correlation ($r = 0.64$, $p < 0.001$) between expression patterns of ABC transporter and IPNS in CuO-NM exposed *F. candida* (Fig. S2†). Furthermore, correlations were found between MTC and IPNS ($r = 0.55$, $p < 0.001$), IPNS and Laminin A ($r = 0.43$, $p = 0.001$) and MTC and ABC transporter ($r = 0.37$, $p = 0.0007$). Finally, weak correlations were found between MTC and Laminin A ($r = 0.28$,

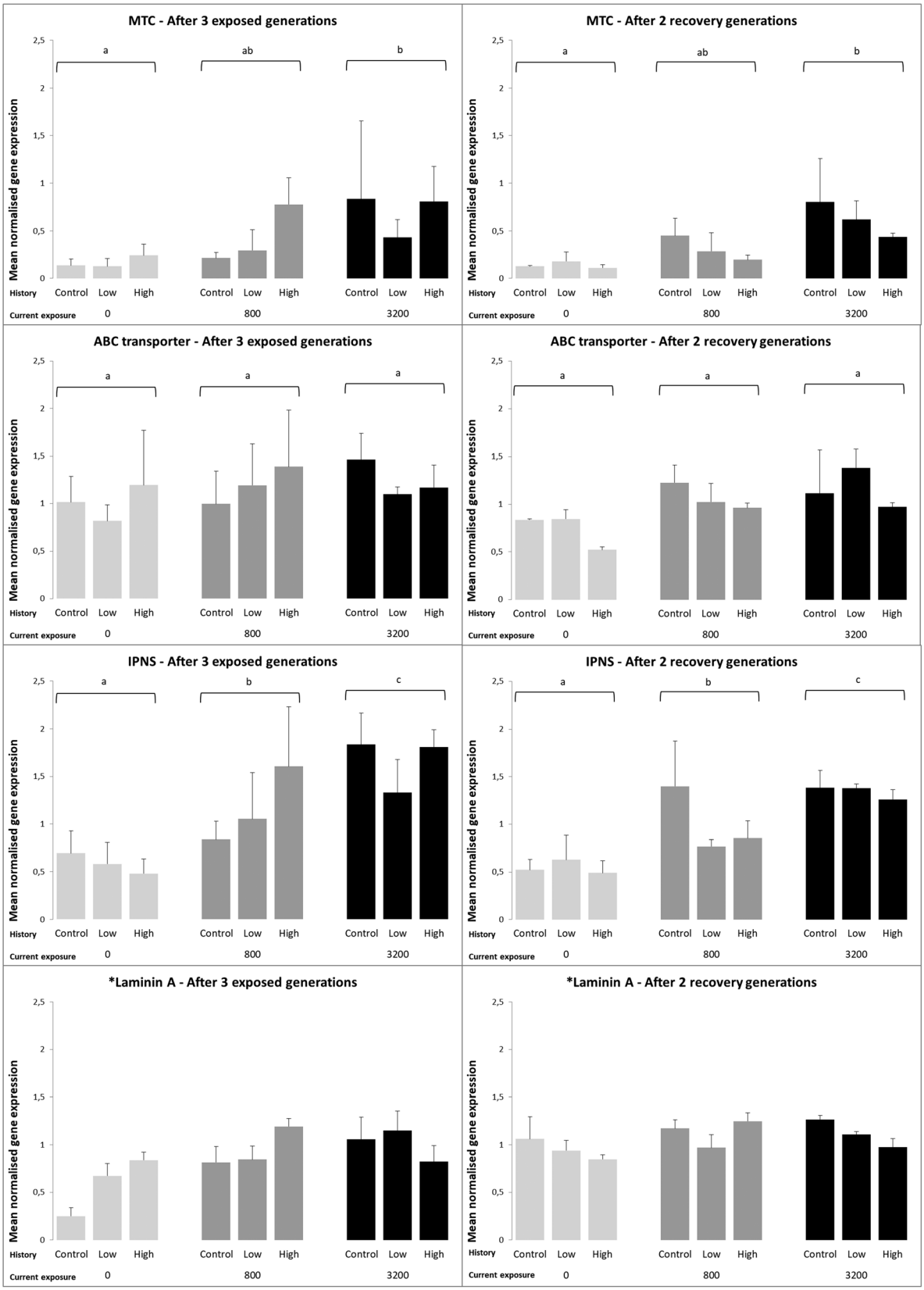

Fig. 6 Effect of CuO nanomaterials on the gene expression of stress response genes in *Folsomia candida* after three exposed generations and after two recovery generations. Nominal historical exposure treatments are provided on the top *x*-axis and current exposure treatments on the lowest *x*-axis. Mean normalized gene expression is plotted on the *y*-axis. Significant effects of current exposures (main effect) are shown above bars. *For Laminin A there were significant interactions (effects of History, Generation and interactions between these three factors). These are described in the text.

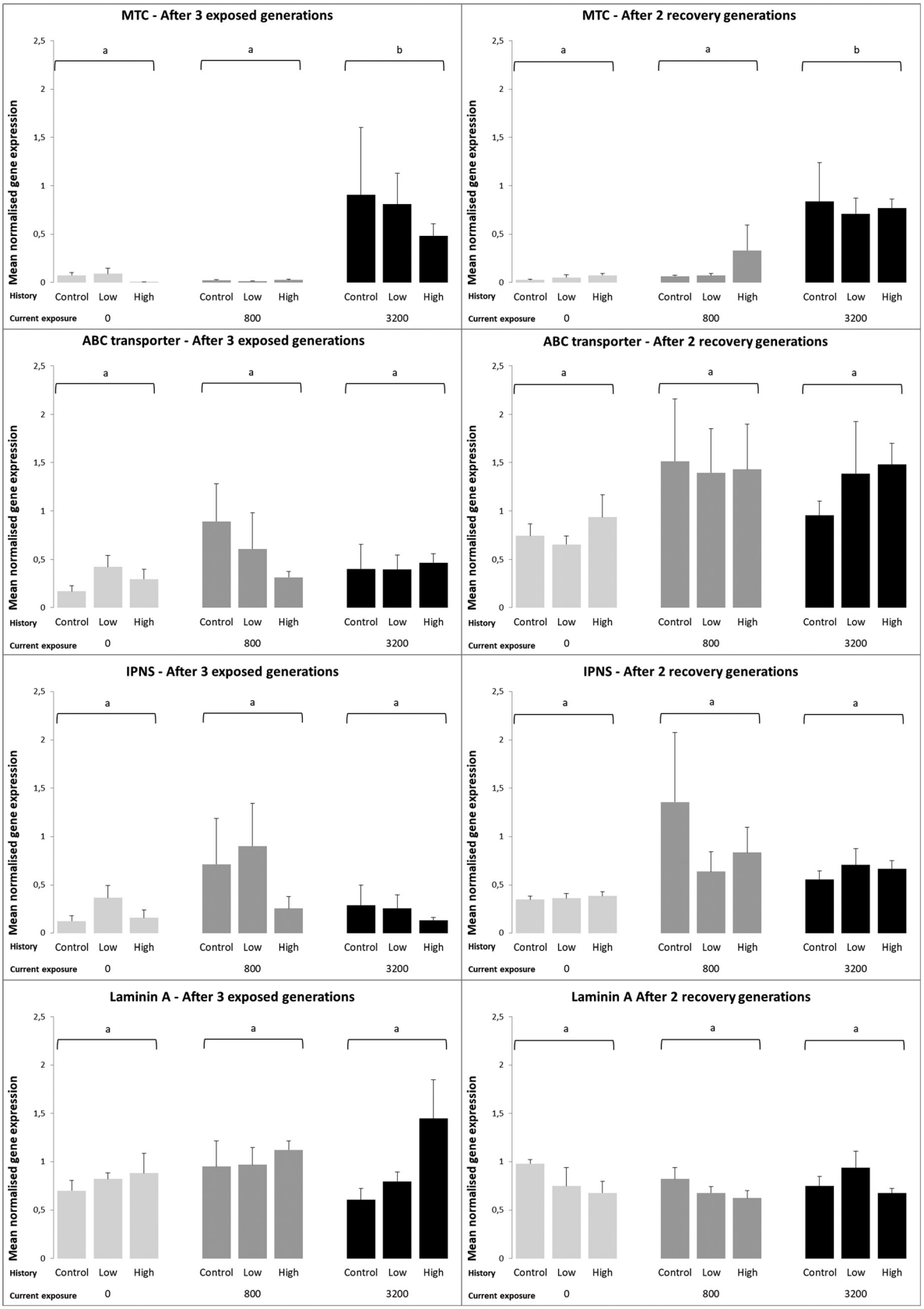

Fig. 7 Effect of WCCo nanomaterials on the gene expression of stress response genes in *Folsomia candida* after three exposed generations and after two recovery generations. Nominal historical exposure treatments are provided on the top *x*-axis and current exposure treatments on the lowest *x*-axis. Mean normalized gene expression is provided on the *y*-axis. Significant effects pf current exposures (main effect) are provided above bars. For ABC transporter and IPNS Generation is also a main effect, which is described in the text.

Table 3 Statistical analysis of the regulation of stress-response genes in *Folsomia candida* exposed to CuO-NM or WCCo-NM over different generations

		Exposure	History	Generation	Exposure: history	Exposure: generation	History: generation	Exposure:history: generation
CuO-NM	MTC	1.122×10^{-6}***	0.48	0.27	0.72	0.99	0.13	0.59
	ABC	0.06	0.80	0.29	0.96	0.75	0.40	0.79
	IPNS	6.277×10^{-7}***	0.64	0.28	0.85	0.80	0.44	0.33
	LAM	0.0002***	0.52	0.001**	0.02*	0.10	0.01*	0.21
WCCo-NM	MTC	$<2 \times 10^{-16}$***	0.98	0.22	0.47	0.12	0.46	0.83
	ABC	0.16	0.65	5.349×10^{-5}***	0.40	0.33	0.98	0.64
	IPNS	0.10	0.97	0.007**	0.29	0.15	0.89	0.42
	LAM	0.86	0.59	0.10	0.35	0.38	0.009**	0.59

$p = 0.04$), as well as between ABC transporter and Laminin A ($r = 0.29$, $p = 0.03$). Exposure treatment affected the strength of correlations between the tested genes. For example, correlation values between ABC transporter and IPNS decreased from $r = 0.75$ ($p < 0.001$) in a control situation, to $r = 0.55$ ($p < 0.05$) in animals exposed to a low concentration, to finally $r = 0.47$ ($p < 0.05$) in animals exposed to a high concentration.

For WCCo-NM exposed *F. candida*, we found that current exposure only had significant effects on the expression of MTC ($p < 0.0001$) (Fig. 7) (Table 3). Very clearly, MTC expression was much higher in all treatments with animals with a high current WCCo exposure level. This pattern is maintained in animals after two recovery generations. Gene expression of both the ABC transporter and IPNS were significantly affected by generation ($F = 21.15$; $F(df) = 1$; $p < 0.0001$ and $F(df) = 1$; $p = 0.007$, respectively) (Table 3). Both genes showed a higher mean expression level in animals after two recovery generations, regardless of history or exposure level. For Laminin A, contrasts analysis on the interaction History*Generation revealed significant differences (Table 3) within the 'High' (3200) history treatment ($F = 3.60$; $p = 0.01$), where animals after two recovery generations showed an on average lower Laminin A gene expression compared to animals that were exposed for three generations (Fig. 7). Correlation analysis of the tested genes in WCCo-NM exposed *F. candida* (Fig. S3†) revealed only one strong significant correlation between the expression of ABC transporter and IPNS genes ($r = 0.89$, $p < 0.0001$). No further correlations between the tested genes were found. The current WCCo treatment of *F. candida* did not affect correlation strength between the expression of ABC transporter and IPNS genes, with $r = 0.89$ ($p < 0.001$) in a control situation and $r = 0.91$ ($p < 0.001$) in both low and high exposure treatments.

4. Discussion

The global impact of manufactured nanomaterials to the environment calls for an assessment of their possible chronic effects on soil ecosystems. The main objective of this study was to determine the phenotypic and transcriptional responses of the springtail *Folsomia candida* following multi-generation exposure to CuO and WCCo nanomaterials (NMs), which are two metal-based NMs commonly used in commercial and industrial applications. We also assessed the potential for recovery after relief from contaminant exposure.

4.1 Ecotoxicological effects of CuO and WCCo nanomaterials

In this study, CuO-NM did not affect phenotypic endpoints such as reproduction and survival of *F. candida* up to concentrations as high as 6400 mg Cu per kg dry soil. Although such concentrations are far above regular environmental concentrations, high concentrations of copper levels may incidentally be found in contaminated soils. For example, Bruus Pedersen *et al.* (2000), reported copper concentrations up to 3000 mg Cu per kg in field soils from Hygum, Denmark, where timber preservation with $CuSO_4$ took place between 1911 and 1974.[27] Kabata-Pendias & Mukherjee (2007) reported concentration up to 7000 mg Cu per kg in soils from polluted areas.[28] Concentrations of cobalt in field soils generally are lower, with values up to 100 mg Co per kg in contaminated soils.[28]

The absence of effects of CuO-NM in the current study could be explained from low porewater metal concentrations, which suggest low solubility or slow solubilisation as was previously described by Noordhoek *et al.* (2018).[23] Previous studies have also reported lower solubility and/or slower dissolution and absence of toxicity for NMs, compared to similar concentrations of readily soluble metal species.[29,30] This was explained by the slow release of metal ions after dissolution of the NMs[1,5] and by aggregation and agglomeration processes, which affect the bioavailability of NMs in the water phase.[31,32] Although in the present study no phenotypic effects of CuO-NM were observed, other studies did report toxic effects of copper NMs to soil invertebrates. For instance, in *Enchytraeus albidus* reproduction was reduced by 50% at 95 mg Cu kg^{-1}.[33] The authors performed a physico-chemical analysis of the particles and found that ions were hardly released by the compound, indicating a NM effect rather than copper toxicity. Bicho *et al.* (2017) performed a reproduction test and a full life-cycle test with *E. crypticus* and found that CuO-NM caused a 10% reduction of reproduction (EC_{10}) at concentrations of 8 and 421 mg Cu kg^{-1}, respectively.[7] The body plan and uptake pathway for potworms (Enchytraeids) is very different from springtails, which could explain in part

the observed differences in NM toxicity. Another point contributing to differences in sensitivity is the variation of internal sequestration and elimination mechanisms between soil invertebrates.[34,35] While Collembola commonly eliminate metals from the gut epithelium by merocrine and holocrine secretion,[36,37] earthworms accumulate them in the chloragogenous tissue surrounding the gut, from which they are eliminated by coelomocytes and the kidneys.[38] These different pathways imply different kinetics and sensitivities.

In the present study, reproduction was not affected in the first two generations exposed to WCCo-NM. We previously already reported that these NMs did not cause any adverse effects on *F. candida* reproduction after 28 days of exposure (Noordhoek *et al.* 2018).[23] Also, the previous study described that porewater concentrations of cobalt were low and not concentration-dependent in WCCo-NM spiked soil, whereas porewater concentrations for soils spiked with cobalt salt showed a concentration-dependent increase with increasing soil metal concentrations. These results indicated lower solubility or slower solubilisation of WCCo-NM than cobalt salts, which may explain the absence of toxicity in the first generation(s). However, in the current study we found a dose-related effect on reproduction in exposed generations 3 and 4 with EC_{50} values of 2463 and 3836 mg Co per kg, respectively. A less profound decline in reproduction was still observed during the recovery phase in recovery generation 1, with an EC_{50} of 5659 mg Co kg^{-1} (based on exposure concentrations in previous generations). Furthermore, WCCo-NM did not affect the survival of *F. candida*, except for animals in recovery generation 1 that were previously exposed to 6400 mg WCCo-NM per kg dry soil. It is unclear why survival was reduced during recovery in clean soil and was not affected when animals were actually exposed to the chemical (the first four generations). Potentially, damage to the gut tissue, as observed in the histological sections (Fig. 5), could lead to a decreased nutritional status of the egg-laying adult. In another springtail, *Orchesella cincta*, Zizzari *et al.* (2016) showed that the quality of the diet has a carry-over effect on maturation time and weight gain of female offspring.[39] Such carry-over effects, acting through energy reserves such as lipids and egg yolk could affect reproduction and survival of offspring for more than one generation, even if the animals are not exposed to the toxic compound anymore, as was the case in recovery generations 1 and 2 in the present study. This is in line with studies reporting tissue damage in other chemical-exposed soil invertebrates. For example, Van der Ploeg *et al.* (2013) found that buckminsterfullerene (C_{60}) exposure induced tissue damage and gene expression alterations in the earthworm *Lumbricus rubellus*.[40]

4.2 Transcriptional responses associated with multi-generation CuO and WCCo NM exposure

The induction of metallothionein (MTC) by both CuO and WCCo NMs is in line with the gene's protective role against metal toxicity.[41,42] Effects on expression on MTC are only present in the current exposure treatments, and expression patterns are not affected by historic exposure. This suggests that MTC is a relevant indicator of direct toxic effect, and less informative for historical multigeneration effects. Yepiskoposyan *et al.* (2006) described induced metallothionein expression in *Drosophila melanogaster* exposed to ionic copper.[41] The authors also observed induced expression of an ABC transporter, suggesting a function in the transport of metal solutes that may aid in elevated resistance to copper. However, it is not known whether the MTC protein of *F. candida* actually binds copper ions; its induction could also be due to alterations in cellular redox status generally caused by copper and nanomaterials.[16] A study by de Boer *et al.* (2011) showed that multiple ABC transporters and copper pumps (copper-transporting ATPase 1, 2) were upregulated in response to cadmium toxicity.[43] In the present study, significantly induced metallothionein expression was found in *F. candida* exposed to either CuO or WCCo NMs and induction of the ABC transporter gene even persisted in animals after 2 recovery generations while in clean soil when previously exposed to WCCo-NM; all indicating a function in maintaining metal or redox homeostasis.

The fact that isopenicillin N synthase (IPNS) gene expression was affected by exposure in CuO-NM treated animals regardless of their exposure history suggests that this gene does not show any alterations over multiple generations and thus indicates direct toxicity. In contrast, IPNS gene expression was significantly affected by generation in WCCo-NM exposed animals, where animals after two recovery generations showed on average a higher IPNS gene expression level compared to animals that were exposed for three generations. *F. candida* is the first animal ever discovered to have beta-lactam biosynthesis genes in its genome,[19,44] with a possible role in maintaining antimicrobial defence against potentially harmful pathogens. The fact that we observed induced IPNS expression in response to CuO exposures is in line with previous studies that reported that the gene is inducible over a wide range of environmental stress factors,[20] suggesting a role for this gene in the general stress response mechanism of *F. candida*. The fact that IPNS gene expression is affected by generation in the WCCo treatment is more puzzling. Such effect should also be expected during CuO exposure, but may have been overruled by the CuO-NM specific effects.

Pearson's correlation analysis revealed a strong correlation between the IPNS and ABC transporter genes in *F. candida* exposed to either CuO or WCCo NMs. This can be explained by the fact that the genes are positioned in the same beta-lactam gene cluster of *F. candida*. A similar co-regulation pattern was also seen in a recent study by Suring *et al.* (2017).[20] They investigated transcriptional regulation of all five genes present in the beta-lactam gene cluster and found that the ABC transporter was co-regulated with IPNS after heat shock treatment. ABC transporters are known to efflux antibiotic compounds out of bacterial and fungal cells in order to attack potential pathogens.[45] Suring *et al.* (2017) speculated about a similar function for this gene in the context of

β-lactam biosynthesis in the springtail.[20] In the present study, the correlation strength between IPNS and ABC transporter decreased by CuO-NM exposure in a dose-related manner. This could indicate a disturbance of the coordination of gene expression due to cellular stress. Interestingly, WCCo-NM exposure treatment did not affect the correlation between IPNS and ABC transporter. Destabilization of co-regulatory networks has been observed in relation to mixture toxicity. For instance, De Boer *et al.* (2013) observed highly fluctuating correlation values among marker genes in their expression response upon exposure to cadmium and phenanthrene in soil.[46] CuO-NM exposures may perhaps cause a mixture of CuO particles and ionic Cu^{2+} molecules leaching from the NMs. Fluctuations between either of the two compounds with increasing exposure concentrations may have caused the increase of variance in gene expression, which could be responsible for a decrease in correlation between the expression profiles of the individual genes. These observations may be highly dependent upon specific NM characteristics.

We also found that Laminin A expression was significantly affected by historical exposures and generation time. This gene encodes a protein that is an integral part of the basal lamina of gut epithelia and other tissues. A previous study showed that metal stress affects cell differentiation and adhesion in the basal lamina in human fibroblasts, where collagen and laminin production decreased upon low-dose exposure to silver and gold nanoparticles.[47] Laminin A gene expression was also affected in cadmium and phenanthrene exposed *F. candida*, irrespective of compound (De Boer *et al.* 2011);[43] suggesting persistent differential gene expression as a result of metal stress. In the present study, a significant effect of the interaction History*Generation on gene expression was found in WCCo-NM exposed *F. candida*. Historical exposure to a high concentration resulted in an induced expression in animals that were exposed for three generations, regardless of the level of current exposure to WCCo-NM. This could indicate that for WCCo-NM, Laminin A is a useful indicator of accumulative stress over multiple generations. Interestingly, this expression profile in animals that were previously exposed to a high concentration was reduced in animals after two recovery generations, while there were no effects of the lower historical exposures. This could indicate that the recovery period is indeed successful in reducing the previously induces stress levels.

The observed transcriptional responses associated with multi-generation exposure to engineered NMs imply that gene expression assays may detect physiological alterations at exposure concentrations that do not show direct effects on reproduction.

Conclusion

The present study revealed adverse effects of WCCo-NM in *F. candida* that appeared only after three generations of exposure. The current study also found induced gut tissue damage in animals exposed to WCCo-NM for four generations, indicating a serious damage to the nutrient absorption function of the gut. This is indicative of carry-over sublethal effects from the parental generation to subsequent generations, possibly through an effect on energy reserves. This could have affected reproduction and survival of offspring for more than one generation, even if the animals were no longer exposed to the compound. Future research should focus on more realistic environmental concentrations, since it has already been shown that especially low concentrations can still have detrimental effects following exposure over a longer periods of time. Gene expression assays detected physiological alterations in response to NM exposure even when consequences on reproduction were not observed. For *F. candida*, both CuO and WCCo NMs caused a significant alteration in expression of stress-related genes. The observed changes in gene expression suggest a metal-specific as well as a general cellular stress response, with the affected genes indicating a function in maintaining cellular homeostasis, metal scavenging and antimicrobial defence. In any case, the present study confirms the additional value of including multi-generation tests and molecular and histological approaches in standardised ecotoxicological testing to help gaining a better understanding of the underlying mechanisms of stress response in soil invertebrates.

Conflicts of interest

The authors declare no competing financial interest.

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